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Biogenesis of the Mycobacterial Cell Wall and the Site of Action of Ethambutol

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The effect of ethambutol (EMB) is primarily on polymerization steps in the biosynthesis of the arabinan component of cell wall arabinogalactan (AG) of *Mycobacterium smegmatis*. Inhibition of the synthesis of the arabinan of lipoarabinomannan (LAM) occurred later, and thus in the cases of AG and LAM, the polymerization of D-arabinofuranose apparently involves separate pathways. While the synthesis of these arabinans was normal in an EMB-resistant isogeneic strain, the addition of EMB to the resistant strain resulted in partial inhibition of the synthesis of the arabinan of LAM and the emergence of a novel, truncated form of LAM, indicating partial susceptibility of the resistant gene(s) and providing a new intermediate in the LAM biosynthetic sequence. A consequence of inhibition of AG arabinan biosynthesis is the lack of new sites for mycolate attachment and thus the channeling of mycolate residues into a variety of free lipids which then accumulate. The primary biochemical effects of EMB can be explained by postulating separate AG and LAM pathways catalyzed by a variety of extramembranous arabinosyl transferases with various degrees of sensitivity to EMB.

The cell wall of *Mycobacterium* spp. is required for growth and survival of the organism in the host (10). The infrastructure, or core, of the cell wall is composed of a covalently linked complex of mycolic acids, the heteropolysaccharide arabinogalactan (AG), and peptidoglycan (15). Much of the primary structure is known from recent (2) and earlier work (16). However, new evidence and concepts have emerged, supportive of the theme of an asymmetric, staggered lipid bilayer within the cell wall, in which the mycolic acids anchored to the arabinan of AG form an inner leaflet and the outer monolayer contains a mixture of phospholipids and members of several classes of glycolipids, depending on the species and/or serotype (4).

One of the most prominent substances intercalating the core cell wall or perhaps anchored in the outer lipid barrier is lipoarabinomannan (LAM) and its simpler version, lipomannan (LM) (6). LAM may have important immunoregulatory functions in tuberculosis and leprosy (1). Disruption of the biosynthesis of any of these components, especially the mycolic acids, AG, or peptidoglycan, should destroy the integrity of the macromolecular assembly. In fact, some of the most effective antituberculosis drugs, isoniazid and ethambutol (EMB), affect mycolic acid and arabinan biosynthesis, respectively (23). The effects of EMB are extremely pleiotropic, and proposals for the primary site of action of EMB have ranged from trehalose dimycolate (TDM) (14) and mycolate (20) metabolism, AG synthesis (21), and glucose metabolism (18) to spermidine biosynthesis (17). However, emphasis is now on inhibition of arabinan biosynthesis, in that Takayama and Kilburn (21) demonstrated that the incorporation of ^{14}C from [^{14}C]glucose into cell wall arabinan of *Mycobacterium smegmatis* was immediately inhibited upon addition of drug to young cultures. Deng et al. (9) demonstrated that this effect applied to the arabinan

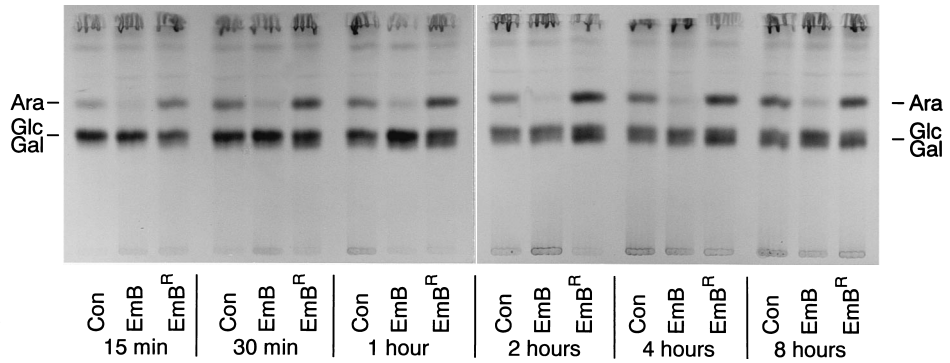

FIG. 1. Effects of EMB on incorporation of ^{14}C from [^{14}C]glucose into sugar components of AG. Delipidated cell pellets from the control (Con), EMB-treated, and EMB-R cultures were extracted with 50% ethanol and 2% SDS in phosphate-buffered saline. The insoluble cell walls were hydrolyzed with 2 M trifluoroacetic acid at 120°C for 3 h, and ca. 50,000 cpm of each extract was applied to cellulose TLC plates. The plates were developed three times in formic acid–water–ethylmethylketone–*tert*-butyl alcohol (15:15:30:40) and exposed to film for 3 days.

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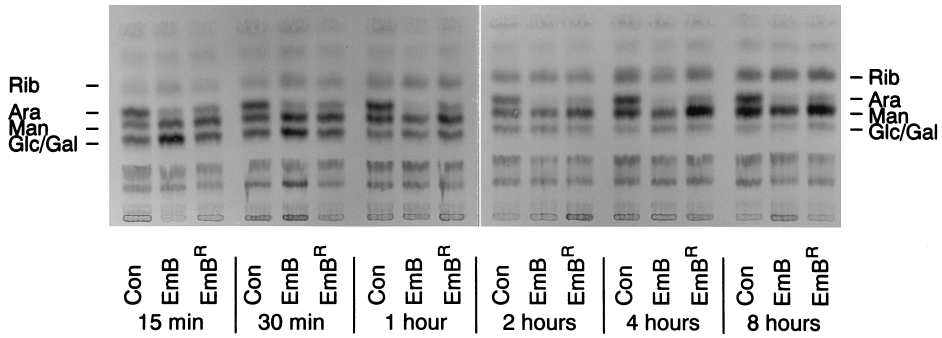

FIG. 2. Effects of EMB on incorporation of ^{14}C from [^{14}C]glucose into sugar components of the LAM-LM fraction of control (Con), EMB-treated, and EMB-R strains. The 50% ethanol extracts were partitioned between phenol and water, the water phases were dialyzed, dried, and hydrolyzed with 2 M trifluoroacetic acid, and ca. 30,000 cpm of each extract was applied to cellulose TLC plates, which were developed as described in the legend to Fig. 1.

of both mycolylarabinogalactan (mAG) and LAM, but in succession. In this study, we have generated EMB-resistant (EMB-R) mutants of *M. smegmatis* which, when examined temporally, allowed important conclusions on the enzymatic targets of EMB, aspects of the differential synthesis of AG and LAM, and the biochemical basis of secondary effects of EMB on mycobacterial metabolism.

MATERIALS AND METHODS

Growth and treatment of *M. smegmatis*. An EMB-R strain of *M. smegmatis* was isolated from the parent strain of *M. smegmatis* mc^2155 (a gift from W. R. Jacobs) by growing initially on plates of 7H11 containing an oleic acid-dextrose supplement (7) and 5 μg of EMB per ml followed by resistant-colony isolation and growth on 10 μg of EMB per ml, as described previously (21). The MIC of EMB for this strain was 50 μg/ml on both broth and solid media. The parent strain of *M. smegmatis* mc^2155 (19), known to be highly sensitive to EMB (0.25 μg/ml on solid medium) was inoculated into 12 flasks, each containing 100 ml of a glycerol-alanine-salts medium. Another six flasks, each containing 10 μg of EMB per ml, were inoculated with the EMB-R strain. After 8 h of growth, EMB was added to half of the flasks containing the control sensitive strain and D-[^{14}C]U-glucose (250 mCi/mmol [9.25 GBq/mmol]; 1 μCi/ml) was added to all flasks. One flask from each of the three sets was removed after 15 and 30 min and 1, 2, 4, and 8 h.

Fractionation of *M. smegmatis*. Cells were harvested by centrifugation, washed with saline, lyophilized, and extracted three times with $CHCl_3$-CH_3OH (2:1) at 50°C for 2 h to yield a lipid-soluble fraction, which was subjected to a partitioning step (11). The lower organic phase contained the total lipid population and was the source of TDM, trehalose monomycolate (TM), and the mycolyl-mannosylphosphopolyprenol (mycolyl-phospholipid; Myc-PL), which were isolated by thin-layer chromatography (TLC) in $CHCl_3$-CH_3OH-NH_4OH (80:20:2) as described previously (3). LAM and LM were removed from the delipidated cells by repeated reflux in 50% aqueous ethanol. These ethanol extracts were combined with the aqueous phase from the lipid extracts, evaporated to dryness, and partitioned between hot phenol and water, resulting in partially purified preparations of LAM-LM (6). The final insoluble material which contained the mycolylarabinogalactan-peptidoglycan (mAGP) complex was further extracted with 2% sodium dodecyl sulfate (SDS) to remove proteins (12). In the case of experiments designed to examine the effects of EMB on mycolate depositions in TDM, TM, Myc-PL, and mAG, control and EMB-treated cells were grown in the presence of 1,2-[^{14}C]acetate (110 mCi/mmol [3.9 GBq/mmol]; 1 μCi/ml) for various periods and fractionated as described above.

Analytical procedures. To estimate the incorporation of radioactivity into individual sugars, fractions from [^{14}C]glucose-labelled cells were hydrolyzed (1 h in the case of LAM-LM, 3 h for AG; 120°C; 2 M CF_3COOH), extracted with $CHCl_3$ to remove fatty acids, and applied to plastic-backed plates of cellulose (Baker-Flex; J. T. Baker, Phillipsburg, N.J.) which were developed three times in formic acid–water–tertiary-butanol–methylethyl ketone (15:15:40:30). Autoradiograms were produced by exposure of the TLC plates to Kodak X-Omat AR film at −70°C. Standards of 200 nmol each of ribose, arabinose, mannose, glucose, and galactose were run in the same fashion and visualized by spraying with a phthalic acid–1-butanol-aniline reagent and subsequent heating at 120°C for 10 min (8). The radioactive monosaccharides released by acid treatment were also assayed by high-performance liquid chromatography with a gradient pump (Dionex, Sunnyvale, Calif.) with pulsed amperometric detection, a Dionex CarboPac PA1 column (4 by 250 mm), and 15 mM NaOH eluent (9). Fractions were collected and counted, and retention times were compared with those of standards. The component [^{14}C]mycolates in TDM, TM, Myc-PL, and mAG from the [^{14}C]acetate-labelled cells were released by saponification with 15% tributyl ammonium hydroxide, converted to the methyl esters, purified by TLC, and counted (22). SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretic transfer of LM and LAM to nitrocellulose were performed as described previously (13). Autoradiograms of the lipid, LAM, and LM profiles from [^{14}C]glucose-labelled cells were obtained by exposing the nitrocellulose to Kodak

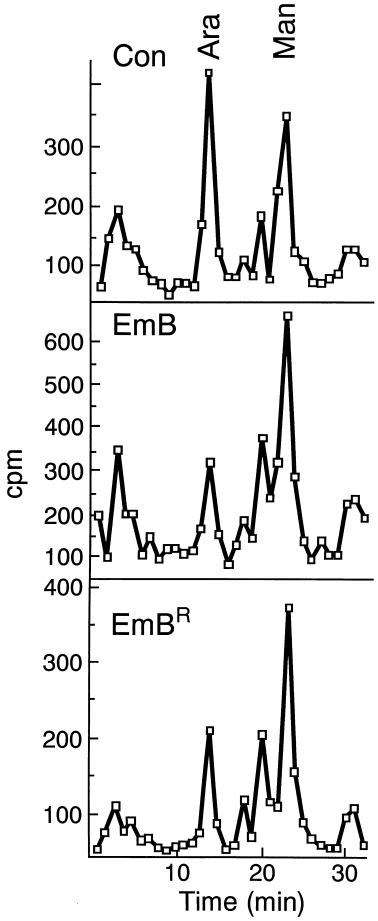

FIG. 3. Effects of EMB on incorporation of ^{14}C from [^{14}C]glucose into sugar components of LAM-LM fractions as determined by high-performance liquid chromatography. The hydrolysates (ca. 6,000 cpm of each) described in the legend to Fig. 2 (those from the 1-h experiment) were applied to a Dionex high-performance liquid chromatography column in conjunction with 30 nmol each of cold Ara, Rib, Glc, Gal, and Man.

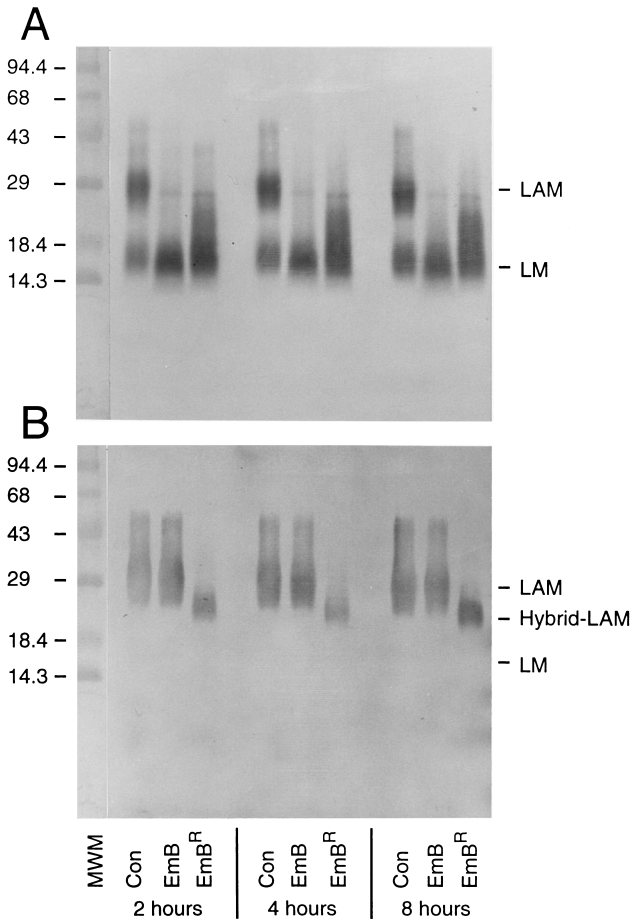

FIG. 4. Effects of EMB on the synthesis and nature of LAM of EMB-treated and EMB-R strains. About 50,000 cpm of each LAM-LM fraction was applied to 13.5% PAGE gels. Blotting to nitrocellulose was performed at 40 V for 3 h. (A) Blots were exposed to film for 3 days. (B) Western blots (immunoblots) were conducted with monoclonal antibody CS-35 (diluted 1:1,000). Con, control.

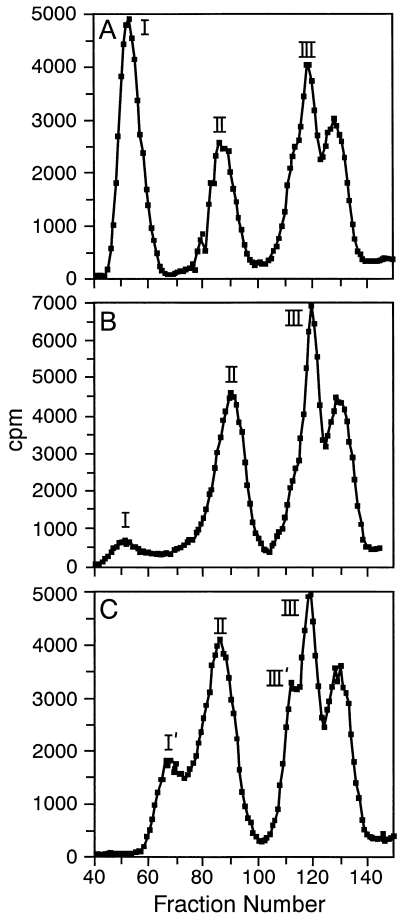

FIG. 5. Gel filtration of the deacylated forms of LAM and LM from control (A), EMB-treated (B), and EMB-R (C) strains. The LAM-LM fractions from the 8-h time points were deacylated in 0.1 N NaOH at 37°C for 2 h and neutralized with 1 M CH_3COOH. Fatty acids were extracted with $CHCl_3$, and the aqueous fraction containing AM and mannan containing ca. 2.5×10^6 cpm were applied to a Bio-Gel P-100 column (1 by 116 cm) in 0.1 M CH_3COOH. Peaks: I, AM; I′, hybrid AG; II, mannan (see text and Fig. 6). Peaks III and III′ were not identified.

X-Omat AR film at −70°C. Immunoblots were obtained as described previously (13). Radioactivity was determined by scintillation counting.

RESULTS

Effect of EMB on synthesis of arabinan of AG. The basic tenet of this communication is that the primary effect of EMB is on the polymerization of D-arabinofuranose. In order to examine the incorporation of ^{14}C from [^{14}C]glucose into AG of the mAGP complex, the final insoluble residues from the fractionated EMB-treated, sensitive control strain and the EMB-R strain were hydrolyzed, chromatographed on cellulose TLC plates, and subjected to autoradiography. A dramatic, rapid, and specific effect of EMB on the incorporation of ^{14}C into the arabinose of arabinan was evident even after 15 min of treatment (Fig. 1), confirming and extending earlier observations (21). EMB had no effect on the synthesis of the galactan component of AG or on the contaminating, insoluble glucans, as evident by normal incorporation of ^{14}C into the galactose and glucose monomers of these polysaccharides (Fig. 1). Also, the EMB-R strain, maintained throughout the experiment on 10 μg of EMB per ml, showed normal arabinan synthesis. From this evidence, it appeared that the primary effect of EMB was on synthesis of the arabinan component of AG.

Effect of EMB on synthesis of arabinan of LAM. One expected a similar effect on the arabinan component of LAM (21), although there are what appear to be minor differences in the detailed structures of the two polymers (5). Fig. 2 is an autoradiogram of the labelled sugars obtained from hydrolysis of the LAM-LM fractions of the sensitive control, EMB-treated, and EMB-R strains. It is obvious that these fractions contained contaminating soluble glucans and ribans, which, incidentally, showed reciprocal labelling patterns throughout the course of the experiment. The effect of EMB on incorporation of ^{14}C into the arabinan of LAM was not definite until after 1 h of exposure, clearly occurring later than the corresponding effect on the arabinan of AG. The differential effect of EMB on the syntheses of the arabinans of AG and LAM suggests that the lesion was not in the early stages of arabinan synthesis but in aspects of final polymerization.

Surprisingly, this late and partial effect of added EMB on the arabinan of LAM from control cells was also evident in the EMB-R cells (Fig. 2 and 3). To examine this effect further, the LAM-LM fractions from the sensitive control, EMB-treated, and EMB-R strains were subjected to SDS-PAGE and autoradiography and subsequently to immunoblotting with the CS-35 monoclonal antibody, which reacts with the arabinan

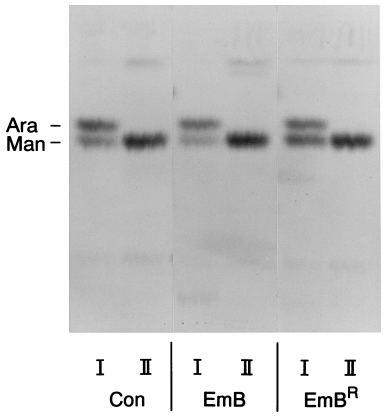

FIG. 6. Natures of the major fractions shown in Fig. 5. Aliquots (ca. 10,000 cpm) were hydrolyzed with 2 M trifluoroacetic acid, applied to cellulose TLC plates, developed three times in formic acid–water–ethylmethylketone–*tert*-butyl alcohol (15:15:30:40), and exposed to film for 3 days.

component of LAM (13) (Fig. 4). The radiolabel patterns (Fig. 4A) again indicated that EMB did inhibit incorporation of ^{14}C into the arabinan of LAM but neither as completely nor as quickly as in the case of the arabinan of AG. This autoradiogram also clearly demonstrated the presence of a product in the EMB-R strain with a mobility intermediate between those of mature LAM and mature LM. The immunoblot (Fig. 4B) clearly demonstrated that this product was a hybrid or a truncated LAM. This immunoblot requires careful study and explanation. Firstly, it was obvious that the antibody reacted only with the arabinan component of LAM, since LM did not react. Secondly, it was obvious that under the saturating conditions applied to this gel, the reaction was not quantitative, since the residual cold LAM present in the EMB-treated cultures prior to the addition of [^{14}C]glucose and EMB did react avidly. With these observations in mind, it was clear that the EMB-R strain of *M. smegmatis* maintained on EMB did not contain mature LAM but instead contained a truncated form. This unexpected result was best seen in a follow-up experiment in which the LAM-LM fraction was deacylated with alkali, converting LAM to arabinomannan (AM) and LM to mannan, and subjected to gel filtration chromatography (Fig. 5). Clearly, at the later times, incorporation of ^{14}C into LAM had ceased. Also, this type of analysis produced much more convincing evidence for the presence of a truncated LAM in the EMB-treated EMB-R strain. When these fractions (i.e., peaks I and I′ in Fig. 5) from the control and EMB-treated cultures were hydrolyzed and subjected to cellulose TLC and autoradiography, it was obvious that they contained arabinose and mannose, and peak II contained only mannose (Fig. 6). Thus, peak I is LAM, peak I′ is a truncated or modified LAM, and peak II is LM, and thus the synthesis of the arabinan of LAM is clearly blocked after 1 h of EMB treatment of the sensitive and EMB-R strains. In a

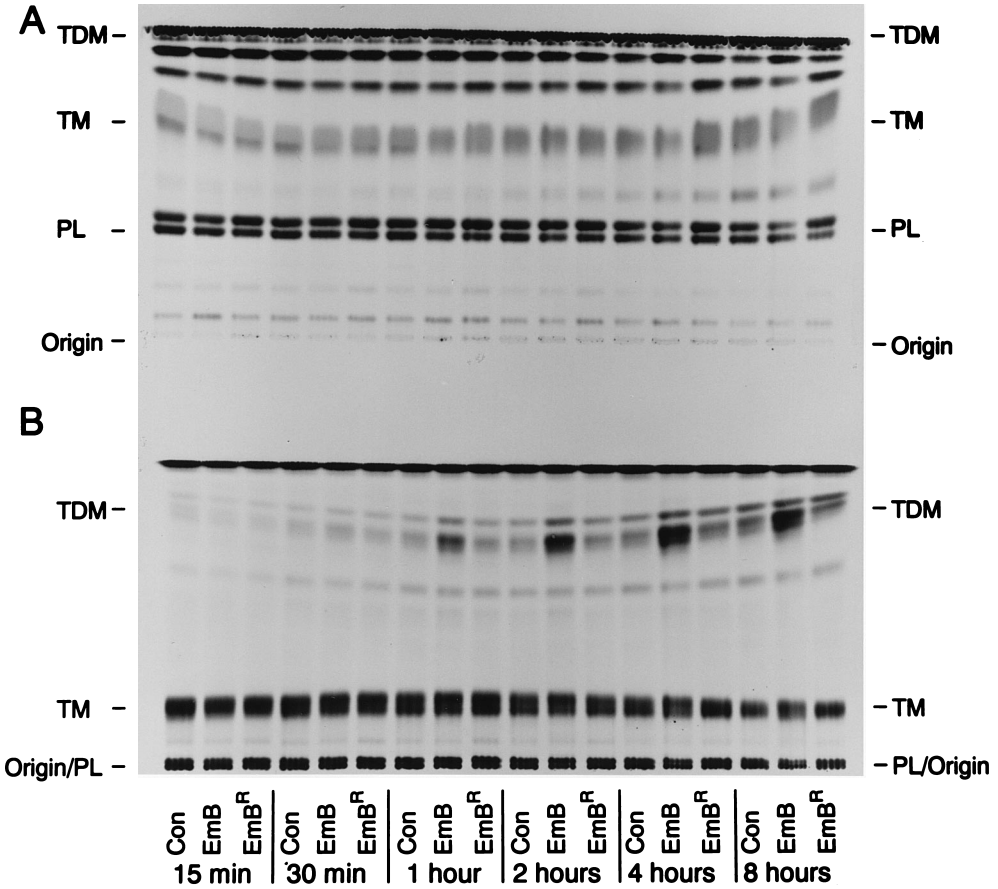

FIG. 7. TLC of the lipids from control (Con), EMB-treated, and EMB-R cultures of *M. smegmatis* labelled with [^{14}C]glucose. Lyophilized cells (dry weight, ~7 to 10 mg) were extracted with chloroform-methanol (2:1), and the washed lipids (ca. 50,000 cpm each) were chromatographed on silica gel TLC plates in chloroform-methanol-water (62:25:4) (A) or chloroform-methanol-concentrated ammonium hydroxide (80:20:2) (B) and exposed to autoradiography film for 5 days.

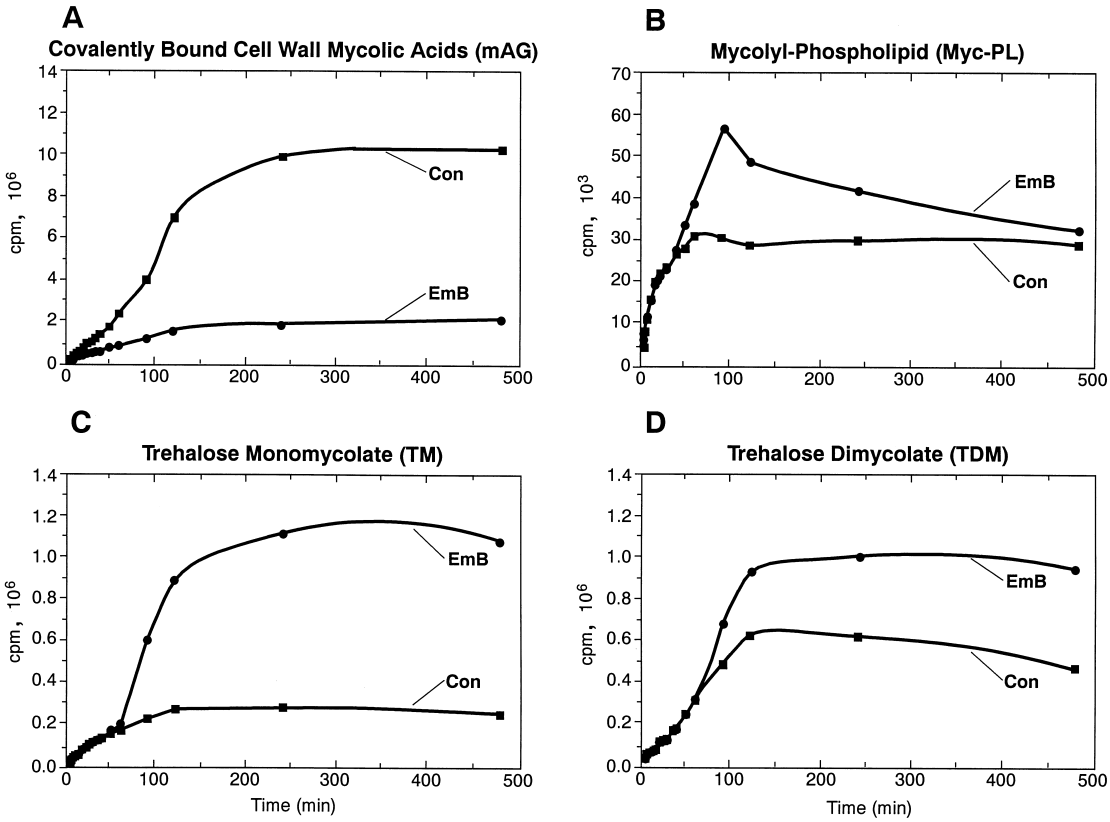

FIG. 8. Effects of EMB on the incorporation of [^{14}C]acetate into the mycolates of the cell wall mAG complex (A), the Myc-PL (B), TM (C), and trehalose dimycolate (D).

subsequent experiment, it was demonstrated that when the EMB-R strain was not maintained in the presence of EMB, then normal wild-type LAM was produced with no evidence of the truncated form, pointing to leakiness in the mutated genes but only in the LAM pathway.

Effect of EMB on incorporation of ^{14}C from [^{14}C]glucose into distinct lipids. Previously, a marked accumulation of radioactivity in TDM under conditions of EMB treatment was observed (14). This observation was placed in perspective in the present experiments. Silica gel TLC of the ^{14}C-labelled lipids from [^{14}C]glucose-labelled cells in a relatively polar solvent showed no effect of EMB on the synthesis of the major classes of extractable lipids (Fig. 7A). However, TLC in a less polar solvent (Fig. 7B) demonstrated marked accumulation of radioactivity in TDM, but not until 1 h of exposure to EMB, a feature which became progressively more obvious at the 2-, 4-, and 8-h time points. Thus, this effect appeared to be secondary to the primary effect on arabinan synthesis.

Effects of EMB on deposition of mycolic acids. If the primary effect of EMB is on synthesis of the arabinan of mAGP, then there should be a diminution of new mycolates in mAGP and a corresponding increase in mycolate incorporation into TDM, TM, and Myc-PL; such a sequala would explain the accumulation of [^{14}C]TDM seen in Fig. 7B. To test this hypothesis, [^{14}C]acetate, which is a selective precursor of the mycolate portions of TDM, TM, Myc-PL, and mAG, was used. A time course experiment showed an immediate inhibition by EMB of [^{14}C]acetate incorporation into the mycolates of mAG (Fig. 8A), followed later by an increase of incorporation of [^{14}C]acetate into the mycolates of Myc-PL (Fig. 8B), TM (Fig. 8C), and TDM (Fig. 8D).

DISCUSSION

Several lines of evidence from the present study and previous studies (9, 21, 24) combine to indicate that the primary site(s) of EMB action is not on de novo synthesis of D-arabinose or on its activation but on its polymerization. We previously demonstrated that one likely precursor of arabinan is β-D-arabinofuranosyl-P-decaprenol (C_{50}-P-D-Ara*f*), and the presence of EMB in cultures of *M. smegmatis* resulted in appreciable accumulation of this product (24), thus indicating that the lesion lay in aspects of its utilization rather than its synthesis. Secondly, the present study indicates that the effects of EMB on the synthesis of the arabinan components of AG and LAM are uncoupled, in that inhibition by EMB of incorporation of ^{14}C from [^{14}C]glucose into the arabinan of AG is complete within 15 min, whereas reaching a similar degree of inhibition in the case of LAM takes 1 to 2 h. If this conclusion is correct (and we realize that there are other explanations for the delayed effect of EMB on LAM synthesis), then it is the terminal, polymerization aspects of arabinan synthesis, specifically the arabinan of AG, that are the first and foremost targets of the drug (Fig. 9). Previously, we noted that "in contrast to cell wall core arabinan, which is completely inhibited by [EMB], synthesis of the arabinan of [LAM] was only partially affected, demonstrating a differential effect on arabinan synthesis in the two locales" (9). We now propose that the syntheses of the arabinans of LAM and AG occur by distinct pathways catalyzed by different arabinosyl transferases, all of which are sensitive to EMB to different degrees. Thus, arabinosyl transferases I and II have lower binding affinities for EMB than does arabinosyl transferase III. It also appears that

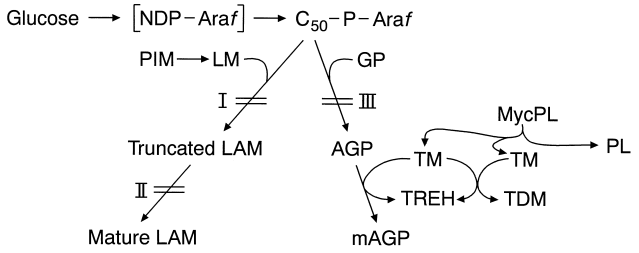

FIG. 9. Schematic drawing of proposed pathways of syntheses of LAM and AG arabinans.

all of these transferases display the resistant phenotype in the case of the EMB-R *M. smegmatis*, with some residual sensitivity to EMB in the arabinosyl transferases involved in the LAM pathway, i.e., arabinosyl transferases I and II, on the basis of the evidence that EMB treatment of the EMB-R strain results in some but not complete inhibition of the synthesis of the arabinan component of LAM, as demonstrated by the fortuitous emergence of a truncated form of LAM. Thus the two- to threefold accumulation of the C_{50}-P-D-Ara*f*, the presumed precursor of all polymerized arabinose in EMB-treated *M. smegmatis* (24), is now readily explained (Fig. 9). Also, the accumulation of TDM, observed previously by Kilburn and Takayama (14) and now observed by us to be a later event (Fig. 7), and also the increased deposition of [^{14}C]mycolates in products other than those that contain arabinan (namely, Myc-PL, TM, and TDM [Fig. 8]), can be explained as essentially tertiary events, the secondary event being lack of [^{14}C]mycolate incorporation into mAG, following the primary event of lack of new arabinan termini on AG. A key product in this scenario, Myc-PL (Fig. 9), was recently isolated from *M. smegmatis* and identified as a 6-*O*-mycolyl-β-D-mannosyl phosphorylpolyprenol (3), and cell-free labelling indicated that it is the means by which mycolates are transported through the cytoplasmic membrane and converted into TM, the presumed direct precursor of arabinan-bound mycolates, i.e., mAG (3). It is probable that TDM or cord factor, like mAGP, is also a metabolic end product. Under conditions in which synthesis of new arabinan within AG had ceased, these alternative mycolic acid-containing products will all accommodate the excess ^{14}C-labelled mycolates (Fig. 9). Incidentally, we have also observed a marked accumulation of LM in EMB-treated *M. smegmatis* (9), which probably arises from inhibition of LAM synthesis, in accord with Fig. 9. Thus, Fig. 9 presents a hypothesis which accounts for many of the known biochemical effects of EMB on sensitive mycobacteria. It now also provides a model for the further application of genetic and biochemical approaches to locating the exact site of action of the drug and to exploring the genetic basis of EMB resistance. Ongoing work comparing the effects of subinhibitory, bacteriostatic, and bactericidal concentrations of EMB should further establish the temporal relationship of these events in the context of growth inhibition followed by loss of viability.

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REFERENCES

1. **Besra, G. S., and D. Chatterjee.** 1994. Lipids and carbohydrates of *Mycobacterium tuberculosis*, p. 285–306. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
2. **Besra, G. S., K.-H. Khoo, M. R. McNeil, A. Dell, H. R. Morris, and P. J. Brennan.** 1995. A new interpretation of the structure of the mycolyl-arabinogalactan complex of *Mycobacterium tuberculosis* as revealed through characterization of oligoglycosyl alditol fragments by fast-atom bombardment mass spectrometry and ^{1}H-nuclear magnetic resonance spectroscopy. Biochemistry **34:**4257–4266.
3. **Besra, G. S., T. Sievert, R. E. Lee, R. A. Slayden, P. J. Brennan, and K. Takayama.** 1994. Identification of the apparent transmembrane carrier in mycolic acid synthesis. Proc. Natl. Acad. Sci. USA **91:**12735–12739.
4. **Brennan, P. J., and H. Nikaido.** 1995. The envelope of mycobacteria. Annu. Rev. Biochem. **64:**29–63.
5. **Chatterjee, D., C. M. Bozic, M. McNeil, and P. J. Brennan.** 1991. Structural features of the arabinan component of the lipoarabinomannan of *Mycobacterium tuberculosis*. J. Biol. Chem. **266:**9652–9660.
6. **Chatterjee, D., K. Lowell, B. Rivoire, M. McNeil, and P. J. Brennan.** 1992. Lipoarabinomannan of *Mycobacterium tuberculosis*: capping with mannosyl residues in some strains. J. Biol. Chem. **267:**6234–6239.
7. **Cohn, M. L., R. F. Waggoner, and J. K. McClatchy.** 1968. The 7H11 media for the cultivation of mycobacteria. Am. Rev. Respir. Dis. **98:**295–296.
8. **Dawson, R. M. C., D. C. Elliott, W. H. Elliott, and K. M. Jones.** 1959. Data for biochemical research, p. 470–475. Clarenden Press, Oxford.
9. **Deng, L., K. Mikušová, K. G. Robuck, M. Scherman, P. J. Brennan, and M. R. McNeil.** 1995. Recognition of multiple effects of ethambutol on the metabolism of the mycobacterial cell envelope. Antimicrob. Agents Chemother. **39:**694–701.
10. **Draper, P.** 1982. The anatomy of mycobacteria, p. 9–52. *In* C. Ratledge and J. Stanford (ed.), The biology of the mycobacteria, vol. 1. Academic Press, London.
11. **Folch, J., M. Lees, and G. H. Sloane-Stanley.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:**497–509.
12. **Hirschfield, G. R., M. McNeil, and P. J. Brennan.** 1990. Peptidoglycan-associated polypeptides of *Mycobacterium tuberculosis*. J. Bacteriol. **172:**1005–1013.
13. **Hunter, S. W., H. Gaylord, and P. J. Brennan.** 1986. Structure and antigenicity of the phosphorylated lipopolysaccharide antigens from the leprosy and tubercle bacilli. J. Biol. Chem. **261:**12345–12351.
14. **Kilburn, J. O., and K. Takayama.** 1981. Effects of ethambutol on accumulation and secretion of trehalose mycolates and free mycolic acids in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **20:**401–404.
15. **Lederer, E.** 1977. The mycobacterial cell wall. Pure Appl. Chem. **25:**135–165.
16. **McNeil, M. R., and P. J. Brennan.** 1991. Structure, function, and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis, and drug resistance; some thoughts and possibilities arising from recent structural information. Res. Microbiol. **142:**451–463.
17. **Paulin, L. G., E. E. Brander, and H. P. Pösö.** 1985. Specific inhibition of spermidine biosynthesis in *Mycobacterium* spp. by the dextro isomer of ethambutol. Antimicrob. Agents Chemother. **28:**157–159.
18. **Silve, G., P. Valero-Guillen, A. Quemard, M.-A. Dupont, M. Daffe, and G. Laneelle.** 1993. Ethambutol inhibition of glucose metabolism in mycobacteria: a possible target of the drug. Antimicrob. Agents Chemother. **37:**1536–1538.
19. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4:**1911–1919.
20. **Takayama, K., E. L. Armstrong, K. A. Kunugi, and J. O. Kilburn.** 1979. Inhibition of ethambutol of mycolic acid transfer into the cell wall of *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **16:**240–242.
21. **Takayama, K., and J. O. Kilburn.** 1989. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **33:**1493–1499.
22. **Wheeler, P. R., G. S. Besra, D. E. Minnikin, and C. Ratledge.** 1993. Stimulation of mycolic acid biosynthesis by incorporation of *cis*-tetracos-5-enoic acid in a cell wall preparation from *Mycobacterium smegmatis*. Biochim. Biophys. Acta **1167:**182–188.
23. **Winder, F. G.** 1982. Mode of action of the antimycobacterial agents and associated aspects of the molecular biology of the mycobacteria, p. 417–521. *In* C. Ratledge and J. Stanford (ed.), The biology of the mycobacteria, vol. 1. Academic Press, London.
24. **Wolucka, B. A., M. R. McNeil, E. de Hoffmann, T. Chojnacki, and P. J. Brennan.** 1994. Recognition of the lipid intermediate for arabinogalactan/arabinomannan biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. J. Biol. Chem. **269:**23328–23335.